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Applicant: LABOFINA S.A. 52, rue de l'Industrie B-1040 Bruxelles (BE)

(7) Inventor: Vandamme, Etienne 62, avenue de la Tenderie B-1170 Bruxelles (BE)

> Schanck-Brodrück, Karin H. 60, rue de la Neuville B-1348 Louvain-La-Neuve (BE)

Colson, Charles 26a, rue des Frères Poels B-1320 Dion Valmont (BE)

Hanotier, Jacques D.V. 5B rue de Caturia B-1338 Lasne (BE)

DNA segment coding for a specific lipase, vectors for the expression thereof, microorganisms transformed by these
 vectors and use of these microorganisms for the production of the lipase.

© DNA segment coding for a lipase which catalyses the hydrolysis of triglycerides with a specificity for fatty acids containing cis-9 unsaturation, characterized in that said segment comprises a sequence selected from the group comprising the following nucleotide sequence:

AGASCTATCCCGTATCTCAGGGCTGGTGCGGGGACAAGCTGTATGCGGTGATTTTTGGGACAAGGGCAAAATTAT
250 260 270 280 290 300 310 320
AACAATGGACCGGTATTGTGCAAAAGGTTTTAGATGAAACGGGTGCGAAAAAAGTGGATATTGTCCCTCG

-410 420 430 440 850 460 470 480 GCGGCGCGGGACAGATCAAAAGATTTTATACACATCATTTAC

490 500 510 520 530 540 550 560 ACCAGTGCCCGATATGCATGATTCATGATTATCATGATGATGATGATGATGATCATGCGTGGAA

 and subfragments and mutants thereof coding for lipases having retained substantially the same specificity.

EP 0 243 338 A

Des ription

DNA SEGMENT CODING FOR A SPECIFIC LIPASE, VECTORS FOR THE EXPRESSION THEREOF, MICROORGANISMS TRANSFORMED BY THESE VECTORS AND USE OF THESE MICROORGANISMS FOR THE PRODUCTION OF THE LIPASE

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TECHNICAL FIELD

The present invention relates to a DNA segment coding for a lipase which catalyses the hydrolysis of triglycerides with a specificity for fatty acids containing cls-9 unsaturation. More particularly, it relates to cloning vectors ensuring expression of said segment in microorganisms and to the microorganisms so transformed by said vectors. It also relates to a process for the production of said lipase by growing such transformed microorganisms.

BACKGROUND OF THE INVENTION

It is well known that the mold Geotrichum candidum produces an extracellular lipase which displays an unique, although not absolute, specificity for fatty acids comprising at least one cis-9 double bond, whatever may be the position occupied by the fatty acid on the triglyceride molecule, i.e. no specificity is observed with respect to the position of the fatty acids within the triglyceride (For a review on the specificity of the G. candidum lipase, see for example R.G. Jensen, Lipids 9, no3, 149, 1974). This specificity may be of interest for industrial purposes since it is known that the severe conditions (230–250°C, 30-40 atmospheres) applied in classical processes to hydrolyse natural fats and oils result in extensive formation of condensation and polymerisation by-products, the more so the starting natural product is richer in unsaturated fatty acids, e.g. soja, sunflower, rapeseed, cartham, linseed oil, etc...

Other practical applications of the lipase from <u>G. candidum</u> may result from another apparently unique property of this enzyme which, in contrast with other lipases, was shown to be able to bring about the esterification of oleic acid with secondary alcohols such as 2-propanol, 3-hexanol or cyclohexanol (S. Okumura et al., Biochim. Biophys. Acta <u>575</u>, 156, 1969. Moreover, it is known that the lipase of <u>G. candidum</u> may find applications in the food industry, particularly as flavouring agent in the dairy industry (M.D.D. Howlett, Biotechnol. in the Food & Drink Ind., First Ntl Conf. Brighton 19-20/9/1983, Conf. Proc. p. 64).

However, these interesting features have so far never been exploited for practical purposes, except for transesterification reactions (Belgian patent no 851,265; 1977). This paradoxical situation probably results from the difficulties inherent to the production of the lipase by the use of <u>G. candidum</u> itself. Not only is this production hampered by the practical problems currently encountered in the large-scale growth of filamentous fungi, but the productivity of <u>G. candidum</u> for the lipase is generally small. As a consequence, producing the lipase necessarily requires co-producing a large amount of biomass, the valorization of which is made difficult by the fact that <u>G. candidum</u> is known to be responsible in men of various pathological manifestations known as geotrichosis (Dictionnaire de Médecine, Flammarion 1982, p. 342).

A way to obviate those difficulties might be to have the lipase produced by the fungus appropriately immobilized. However, this method is made unpractical in the present case by the fact that the production of lipase by <u>G. candidum</u> strictly requires aerobic conditions (Y. Tsujisaka et al., Agr. Biol. Chem. <u>37</u>, no4,837, 1973) and the presence of lipids M. Iwai et al., Agr. Biol. Chem. <u>37</u>, no 4, 929, 1973) making the medium a complex polyphasic system.

SUMMARY OF THE INVENTION

It is the main object of the present invention to make the lipase of <u>G. candidum</u>, or any variant thereof having retained substantially the same specificity, available for industrial applications by having it produced by a selected microorganism genetically engineered for this purpose.

This object is accomplished by providing a DNA segment coding for a lipase catalyzing the hydrolysis of triglycerides with such a specificity that fatty acids comprising at least one cis-9 double bond are selectively liberated, said segment comprising a sequence being selected in the group consisting of the following sequence and the subfragments and mutants thereof coding for lipases having retained substantially the same specificity.

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10 ATGAAATTTGTAA								
ATGAAATTTGTAA	20	30	40	50	60	70	80	
	AAAGAAGGATO	ATTGCACTT	GTAACAATTT	GAT GCT GTC	TGTTACATCG	CTGTTTGCGT	TGCAGCC	
			•					
90	100	110	120	130	140	150	160	5
6T CAGCAAAAGC C	SC I GAACACA	TICCAGICGT	TATEGTTCAC	SGTATTGGAG	GGGCACCATTI	CAATTTTGCG	GGAACTA	
170	180	190	200	210	220	230	240	
AGAGCTATCCCGT							240	
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250	260	270	280	290	300	310	320	
AACAATGGACCGG	TATTATCGCGA	TTTGTGCAA	AAGGTTTTAG	TGAAAC GGG	TGCGAAAAAA			
								15
330	340	350	360	370	.380	390	400	15
CAGCATGGGGGGC	GCGAACACACT	TTÄČTACAT	AAAAATCTG	SACGGCGGAA	ATAAAGTTGC	AAACGT CGT G/	CGCTTG	
	400							
-4-10	420 ETTCACGACAC	430	440	450	460	470	480	20
GCGGCGCGAACCG	IIIGACGACAG	BLAAGGLGL	I I C C G G G A A C /	NGA I CLAAA I I	CARAGAIII	IAIACACAIC	CATTTAC	20
490	500	510 .	520	530	540	550	560	
AGCAGTGCCGATA								
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570	580	590	600	610	620	630	640	
CATCGGCCTTCTG	TACAGCAGCCA	AGTCAACAG	CTGATTAAA					
						•		
								<i>30</i>
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Table. Amino acid composition of the lipase of $\underline{G.candidum}$ (in mole %)

Amino acid	From	New	From nucleotide
	Tsusijaka et al.	determination	sequence
Trp	1.8	1.8*	0.9
Lys	3.8	4.0	6.6
His	2.4	2.6	1.9
Arg	3.7	3.9	3.8
Asp + Asn	. 13.1	14.8	12.3
Thr	5.2	5.5	6.1
Ser	6.8	7.7	7.1
Glu + Gln	7.4	7.7	4.7
Pro	6.5	3.0	3.3
Gly	9.8	9.5	11.3
Ala,	8.2	8.2	7.6
Cys	0.0	0.0	0.0
Val	6.0	5.2	9.4
Met	0.0	2.1	2.8
Ile	4.3	3.9	5.7
Leu	9.9	9.6	9.4
Tyr	4.5	4.8	4.3
Phe	6.6	5.7	2.8

^{*} assuming the same value as determined by Tsujisaka et al.

The discrepancies between the composition as calculated from the nucleotide sequence given hereabove

and the analytical data obtained from actual preparations of the lipase can in part be ascribed to insufficient purification of the enzyme but also to the fact that the lipase is normally secreted by G. candidum (W.O. Nelson, J. Dairy Sci. 35, 455, 1952). Therefore, the polypeptide encoded by the lipase gene should comprise a signal sequence allowing transport of the lipase into endoplasmic reticulum and which is cleaved off in the mature protein. However, as it will be shown hereinafter, homogenates prepared from E.coli wherein the lipase gene has been expressed as an intracellular protein display lipase activity with the same specificity as the mature protein secreted by G. candidum. It is to be understood that the present invention not only relates to the original gene whose nucleotide sequence is given hereabove but also to all variants obtained therefrom by mutation and/or deletion and coding for polypeptides having retained at least partially the typical specificity of the G. candidum lipase. Specifically, the DNA region coding for the mature lipase as it is actually secreted by the mold has to be considered as comprised within the scope of the present invention. Similarly, should be considered as also comprised in the invention any construction wherein said region would be fused with another DNA fragment coding for a signal sequence allowing secretion of the lipase from the microorganism selected as host. For example, when yeasts are to be used for the production of heterologous polypeptides, it is current practice to fuse the DNA region coding for this polypeptide with sequences encoding the leader region of the precursor of the yeast mating pherormone α-factor (see for example A.J. Brake et al., Proc. Natl. Acad. Sci. USA 81, 4642, 1984). Another possibility is to fuse the interesting coding region with the DNA fragment coding for an heterologous signal sequence which is recognised by the yeast cleavage mechanism, e.g. the signal sequence of chicken lysozyme (Belgian Patent no 901,223; 1985). Such constructions aiming at ensuring secretion by yeast of a polypeptide having substantially the same lipolytic activity and specificity as the G. candidum lipase have to be comprised within the scope of the present invention.

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According to another aspect of the present invention, there are provided the cloning vectors for ensuring expression of the lipase-coding DNA segment as hereabove defined. These vectors have obviously to be selected in consideration of the recipient microorganism chosen. When E.coli is preferred, classical multicopy plasmids as e.g. those derived from the endogenous replicons CoIE1, pMB1 and p15A, and particularly those of the pBR322 series will be used with advantage.

Other bacterial hosts of the genus Bacillus, Pseudomonas, Streptomyces, ... can also be used for cloning the lipase-coding segment. However, according to a preferred embodiment of the invention, this cloning will be carried out in yeast. As those skilled in the art know, yeasts have various practical advantages over bacteria for industrial purposes, especially for food and feed applications. Not only are several yeast species GRAS, but they are also a valuable source of proteins and vitamins for feed formulations. Moreover, they are especially suitable for large-scale fermentations, being not susceptible to phage infection and requiring only semi-sterile conditions. Another advantage of yeasts as industrial microorganisms for the production of polypeptides is that in many cases they are amenable through genetic engineering to protein excretion so that the desired polypeptide can be readily recovered from the culture medium by classical down-stream processing means such as chromatography, ultrafiltration, precipitation, etc... Still another advantage of yeasts result from the fact that they can be grown under anaerobic conditions, this making easier their use in immobilized form for the production of metabolites, including enzymes. For such purposes, the yeast Saccharomyces cerevisiae whose molecular biology is especially well known and which has been used since immemorial times in practical food applications is especially suitable. However, other species of yeast transformable by exogenous DNA can also be used. As examples of such other yeasts, one may cite Saccharomycopsis lipolytica, Schizosaccharomyces pombe, Kluyveromyces lactis, Candida cylindracea, etc... Some of these species are more prone than S. cerevisiae to excrete proteins into the culture medium. That is the case, for example, of Candida cylindracea which is well known to secrete a lipase into the culture medium from which the enzyme can be recovered, e.g. by precipitation (U.S. patent no3,189,529; 1965). Saccharomycopsis lypolytica is also known to excrete a lipase while two other lipases are secreted but remain associated with the cell wall (Y. Ota et al., Agr. Biol. Chem. 46, no 12, 2885, 1982).

However, as known by those skilled in the art, it is especially with yeasts of the genus Saccharomyces and still more especially with those strains belonging to the species S. cerevisiae that cloning techniques ensuring the production in substantial amounts of heterologous proteins are the most efficient, allowing in some cases exceptionnally high expression levels, e.g. for superoxide dismutase (International application WO 85/01503). To ensure in S. cerevisiae the expression of the lipase-coding DNA segment of the present invention, different kinds of cloning vectors are available. Integration vectors can be used for this purpose; however according to a preferred embodiment of the invention, cloning will be achieved by resorting to autonomously-replicating plasmids. Such plasmids comprise the replication origin of the 2-micron endogenous plasmid present in most strains of these species or, eventually, an ars segment of autonomous replication of chromosomial origin. Such plasmid must also contain a marker gene allowing visualization and selection of the cells which have effectively been transformed with the plasmid. As a marker gene, there is generally used a gene which codes for an enzyme involved in the biosynthesis of an essential metabolite, e.g., an amino acid. In such a case, the host cell to be used is a yeast strain which, through mutation, has become auxotrophic for this metabolite. By inoculating with this strain a medium free from said metabolite, only those cells transformed by a plasmid bearing the missing gene will be able to grow. Typical examples of such markers are the genes LEU2 and TRP1 which respectively code for an enzyme envolved in the biosynthesis of leucine and tryptophane. Another typical example is the gene URA3 which codes for an enzyme involved in the biosynthesis of uracil. These expression vectors must also comprise one, or preferably several unique restriction sites for insertion of the

coding part of interest, as well as the various elements required for optimizing the expression thereof, i.e., promoters, terminators, and other control elements.

These yeast vector plasmids often further comprise bacterial sequences capable of ensuring their replication and their selection in an intermediate bacterial host, e.g. E.coli. As classical examples of such shuttle plasmids, one may cite YEp13 (J.R. Broach et al., Gene 8, 1979, 121) pFL1-4 (M.R. Chevallier et al., Gene 11, 1980, 11), pJDB207 (J.D. Beggs Alfred Benson Symposium no 16, Munksgaard, Copenhaegen, 1981, p. 383), pHM158 and pJO158 (M. Heusterspreute et al., Gene, 34, 1985, 363-366).

According to a preferred embodiment of the invention, a plasmid comprising at least the replication (REP) functions of the sequence of the 2-micron endogenous plasmid is used, mainly when the host cell belongs to the S. cerevisiae species. Said functions generally bring to the plasmid a greater stability, particularly if the host cell has beforehand been cured of its 2-micron plasmids (C.P. Hollenberg, Curr. Top. Microbiol. Immunol. 96, 1982, 119; R.M. Walmsley et al., Mol. Gen. Genet. 1983, 361). Classical examples of such vectors are plasmids pJDB219 and pJDB248. (J.D. Beggs, Nature 275, 1978, 104).

Finally, to ensure an expression level as high as possible of the coding part of interest, it is necessary to associate it with a promoter as efficient as possible. Various strong promoters are known in yeast, e.g., the promoters of alcohol dehydrogenase (ADH1), enolase (ENO8 and ENO46), glyceraldehyde-3-phosphate dehydrogenase (GAP63 and GAP491), phosphoglycerate kinase (PGK) (M.J. Dobson et al., Nucleic Acids Res. 10, 1982, 2625), alkaline phosphatase (PH03 and PH05) (European patent application no100,561) or still the promoter p415 or the variants thereof (Belgian patent no901,222 1985).

These various techniques, which have been successfully applied to the cloning and the expression of many heterologous genes in yeast, can be used as well for ensuring in yeast expression of the lipase-coding segment of the present invention. Examples of specific constructions performed according to standard methodologies (T. Maniatis et al., Molecular Cloning, Cold Spring Harbor Lab., 1982) are given in the present patent for a purpose of illustration but it is evident that other possibilities exist and that various replication origins, marker genes, efficient promoters and other structural elements may be combined to obtain similar results.

In accordance with another aspect of the invention, the transformed cells obtained in those various cases must also be considered as being within the scope of the invention.

When a microorganism, bacterium or yeast, has been induced according to the present invention to produce a lipase displaying the desired specificity for unsaturated substrates, it is necessary to grow it under the most favourable conditions to its growth in order to take advantage of this new trait. One skilled in the art will easily determine these conditions according to the characteristics peculiar to the microorganisms used as host

As transformed microorganisms have in most cases a more or less marked tendency to lose artificially-constructed plasmids, it is advantageous to use such a culture medium as to exert a positive selection pressure on them. When the recipient yeast is a mutant auxotrophic for one or another essential metabolite and when the vector plasmid used comprises a marker gene capable of restoring prototrophy to the strain, e.g., the LEU2 or TRP1 genes mentioned above, this selection pressure may be exerted by omitting said metabolite from the culture medium. On the contrary, if the plasmid comprises as marker a gene capable of conferring to the yeast a more or less marked resistance to a growth inhibitor, e.g., an antibiotic such as G418 (J. Jimenez and J. Davies, Nature 287, 1980, 869) or an herbicide such as diuron (Belgian patent no 899,607; 1984), the selection pressure in favor of the transformed yeast can be applied by growing it in a medium supplemented with this inhibitor. Other means exist to obtain the same result and may also be used to practice the invention.

When transformed yeasts have been grown under conditions ensuring the best production of the desired lipase, this has still to be recovered. Many techniques are available which those skilled in the art will combine to obtain in each case the best recovery yield and the greatest purity of the lipase. However, according to a preferred embodiment, the DNA region coding for said lipase will preferably be equipped with a leader sequence coding for a signal peptide capable of ensuring the transport of the lipase through the plasmic membrane of the transformed cell. In this case, the separation of the enzyme will indeed be considerably easier, whether it is liberated into the medium from where it will be recovered by classical methods or it remains associated to the yeast cell wall from which it will have to be separated by other methods.

The various aspects of the present invention will appear more specifically in the following examples which are purely illustrative and should not be construed to limit the scope of the invention.

EXAMPLES

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1. Cloning in E.coli of the lipase-coding segment, using bacteriophage λNM590 as insertion vector
A DNA preparation from phage λNM590 (N.E. Murray et al., Mol. Gen. Genet. 150, 53, 1977) and another
DNA preparation from the mold G.candidum were both cleaved with restriction endonuclease HindIII. Phage
λNM590 has an unique HindIII restriction site and the two fragments generated therefrom were ligated, using
T4 DNA ligase, with an equivalent amount of DNA fragments from G.candidum. The recombinant DNA thus
obtained was then packaged by mixing with proteins prepared by the method of B. Hohn and K. Murray (Proc.
Natl. Acad. Sci. 74, no8, 3259, 1977) from the defective phages present in the two E.coli strains BH2671 and
BH2673. Complete phage particles thus obtained were then used to infect E.coli strain HB101.

Seven Petri dishes containing "Spirit Blue Agar" (Difco) and "lipase reagent" (Difco) were inoculated with infected bacteria and incubated overnight at 37°C whereby approximately 1000 plaques of lyse appeared per Petri dish.

One of these plaques showed lipase activity as evidenced by the development of a typical blue coloration. The phage responsible for this activity and called $\lambda NM590$ lip was purified by dilution and prepared in relatively large amount by lysing in liquid medium <u>E.coli</u> strain CL1204 (C600 <u>leu serB thi lac hsdR</u>) known to be easily infected by $\lambda phages$. The phages were then separated and further purified by ultracentrifugation on preformed CsCl gradient and dialysis against Tris buffer pH 7.5.

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The DNA of the phages thus purified was separated by double phenolisation and dialysis against Tris/EDTA buffer pH 8. Upon characterization by restriction analysis, it was determined that the lipase-coding region was associated with a 3.9 kb Hindlll insert comprising itself two Hindlll subfragments of 2.3 and 1.6 kb respectively.

2. Cloning in E.coli of the lipase-coding segment, using plasmid pBR322 as vector

The fragments obtained by incomplete digestion with restriction enzyme Hindill of a DNA preparation from phageλMN590lip obtained as described in the preceding example was ligated with pBR322 opened with the same enzyme and dephosphorylated by alcaline phosphatase. The recombinant DNA thus obtained was used to transform E.coli strain HB101 (leu pro lac thi rpsl_recA hsdS; H.W. Boyer & D. Roulland-Dussoix, J. Mol. Biol. 41, 459, 1969) made competent by CaCl₂ treatment and the transformants were then screened for lipase activity by transfer on Petri dishes of "Sprit blue Agar" with "lipase reagent": 8 clones were shown to be positive by the development of a blue halo around the colonies.

One of these positive clones was chosen for efficient lipase production and was shown to be tetracycline sensitive. This clone will be referred to hereinafter as pLIP1. The plasmid contained therein was characterized by restriction analysis followed by electrophoresis on agarose gel and was shown to contain a 2.3 kb insert. Through further characterization by electrophoresis on acrylamide gels, it was determined that this insert comprises three EcoRI sites giving rise through restriction to one relatively large HindIII-EcoRI fragment of about 1400 bp and three smaller fragments of about 520, 230 and 120 bp as shown on Figure I.

In another construction, the large and the small external HindIII-EcoRI fragments were separated and ligated through a triple ligation event with plasmid pBR322 open with restriction enzyme Hind III and the resultant recombinant plasmid used to transform HB101. The transformants thus obtained displayed lipase activity which shows that the lipase-coding region is entirely comprised within the larger HindIII-EcoRI fragment. Figure II shows the nucleotide sequence (as determined by the method of A. Maxam and W. Gilbert, Methods in Enzymology, Ed. by L. Grossman and K. Moldave, Acad. Press. inc., New York & London, Vol. 65, p.499, 1980) of a DNA segment comprising said larger fragment. It can be seen that it contains only one open reading frame coding for a protein of more than 200 amino acids: this coding region starts with an ATG initiation codon on position 203 and ends after position 838 by a double stop signal (TAA, TGA).

Moreover, the 5' flanking region thereof comprises a typical Pribnow box TATAAT 38 bp upstream from the ATG and a typical Shine-Dalgarno sequence AGGAGG starting 11 bp upstream from the ATG.

Further evidence that this coding region actually corresponds to the lipase produced by the bacteria transformed with pLIP1 plasmid was gained by the following deletion experiments: (a) plasmid pLIP1 was cleaved at its unique Clal and Ncol sites to generate a 5800 bp fragment and a 733 bp fragment comprising the 5' end of the coding region containing itself two Hpall restriction sites; (b) the smaller fragment was cleaved with Hpall; (c) the Clal-Hpall (434 bp) and the Hpall-Ncol (110 bp) fragments generated by the latter restriction and the 5800 bp Clal-Ncol fragment generated by the former were purified and simultaneously ligated to give a plasmid deleted from a 186 bp Hpall-Hpall fragment within the coding region; (d) this plasmid was used to transform E.coli. No lipase activity was detected in the resulting transformants.

Finally, to ascertain that the lipase produced by the bacteria transformed with plasmid pLIP1 has the desired specificity displayed by the lipase from G.candidum, a crude enzyme preparation obtained by sonication and lyophylisation of transformed bacteria was used to hydrolyse an equimolecular mixture of triolein and tripalmitin according to the method of Cl. Franzke et al. (Die Nahrung 17, no2, 171, 1973). The free acids liberated by action of the lipase were separated by thin-layer chromatography using as solvent a mixture petroleum ether/diethylether/acetic acid 70/30/2, transformed into methyl esters by the method of P.A. Biondi & M. Cagnasso (J. Chromatography 109, 389, 1975) and analyzed by vapour-phase chromatography using a 10% Silar 5CP/chromosorb WHP 80/100 mesh (Chromopack) column. The proportion of free acids thus determined was in mole %: palmitic acid, 8%; oleic acid, 92%.

Strain E.coli HB101(pLIP1) has been deposited on April 22, 1986 at the Centraal Bureau voor Schimmelcultures, Oosterstraat 1, P.O. Box 273, NL - 3740 AG Baarn (The Netherlands), where it has been given accession number C.B.S. 229.86.

3. Cloning of the lipase-coding segment in yeast

3.1. Insertion of the 2.3 kb Hindlll fragment in vector pJDB207

The 2.3 kb Hindlll DNA fragment responsible for lipase activity of bacteria transformed with plasmid pLIP1 as described in the preceding example was inserted in a Hindlll site of the yeast-E.coli shuttle plasmid pJDB207 (J.D. Beggs, Genetic Engineering 2, Ed. by R. Williamson, Academic Press 1981, p. 175) containing the bacterial ampr and tetr genes imparting resistance to ampicillin and tetracycline for selection in E.coli as well as

the yeast LEU2 gene for selection in Leu-strains of yeast. The resulting recombinant plasmids were then used to transform E.coli strain HB101 and the transformants were screened for ampicillin resistance. Upon transfer of 100 resistant clones on "Spirit blue Agar", 9 of them were shown to b lipase-positive; they were also shown to be tetracyclin sensitive. One of these clones called pEK8 was selected for transformation into yeast. Plasmid DNA from this clone was isolated by the method of H.C. Birnboim & J. Doly (Nucleic Acid Res.7, no6, 1513, 1979) and us d to transform yeast strain AH22 (leu 2-3 leu 2-112 his 4-519 CAN). Transformants were screened for leucine prototrophy on minimal medium (0.67% yeast nitrogen base, 2% glucose) supplemented with 0.002% histidine, 1M sorbitol and 2% agar. The resulting transformants were then tested for lipase activity by culturing in liquid minimal medium supplemented with histidine, disrupting the cells in phosphate buffer 0.1M pH 7 by shaking with glass beads in a Braun homogeneizer, and determining lipase activity by a radioassay adapted from the method of M.C. Scholtz et al. (J. Lip. Res. 11, 68, 1970). No significant lipase activity could be detected in the cell homogenate. To ascertain that the lipase-coding segment was actually present in the transformed yeasts, the plasmid DNA present therein was extracted and used again to transform E.coli strain HB101. The transformed clones were shown to be lipase-positive. The conclusion was that the control elements accompanying the lipase-coding region in the original 2.3 kb fragment from the G.candidum genome are correctly recognized by E.coli but not by the yeast expression

3.2. Cloning of the lipase-coding segment in S.cerevisiae, using expression vector pEX-2

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In order to ensure expression in yeast of the lipase-coding segment of the present invention, it appeared necessary, in view of the preceding results, to delete the 5' flanking region thereof, upstream from the ATG initiation codon, and then to fuse in an appropriate yeast expression vector the trimmed fragment with a yeast promoter. In this construction, the yeast-E.coli shuttle plasmid pEX-2 was selected as expression vector for its ability to ensure expression of heterologous genes as well in E.coli as in yeast (J.F. Ernst and R.K. Chan, J. Bacteriol. 163, no1, 8, 1985). This plasmid comprises the ampr marker gene for selection in E.coli and the URA3 marker gene for selection in Ura- strains of yeasts. The different steps of the construction were the following (see Fig. III):

a) Plasmid pLIP1 was opened at its unique <u>Clal</u> site and digested at 20oC for 1.30 min. with nuclease <u>BAL31</u> (0.1 units/μg of DNA), the latter reaction being stopped by phenolisation.

b) The population of shortened plasmids thus obtained were then recirculized using a linker BamHI 12 MER inserted by the method of R. Lathe et al. (DNA 3, 173, 1984) and transformed into E.coll strain BJ5183 (F-recBC sbcB endol gal met str thi bio hsdR; M.R. Chevallier, Mol. & Cell. Biol. 2 no8, 977, 1982) remarkable for its high transformation frequency.

c) From 960 transformed clones, 613 (64%) had lost lipase activity. These inactive clones were pooled for growth in selective medium (1% tryptone, 1% NaCl, 0.5% yeast extract, 50 mg/l ampicillin) and isolation of plasmid DNA.

d) The plasmid DNA thus prepared was cleaved with restriction enzyme <u>BamHI</u> and the resulting fragments separated by electrophoresis on agarose gel. Those fragments bearing the lipase-coding region migrated as a single large band which was recovered from the gel on DEAE-cellulose paper by the method of G. Dretzen et al. (Anal. Biochem. <u>112</u>, 295, 1981).

e) The fragments thus isolated were then ligated with plasmid pEX-2 opened at its unique BamHI site between the CYC1 promoter and terminator regions and dephosphorylated by action of bacterial alcaline phosphatase (Worthington).

f) The population of recombinant plasmids thus obtained were transformed into E.coli strain BJ5183 and transformants simultaneously screened for ampicillin resistance and lipase activity: 26 clones out of about 25000 were selected for their ability to develop lipase activity within 2 days as evidenced by the blue color developed on "Sprit blue Agar" supplemented with "lipase reagent" and with ampicillin for selection of transformants.

g) The plasmid DNA from these lipase-positive clones was isolated by the method of Bimboim & Doly (ref. cit.), and used to transform E.coli strain HB101 more appropriate than strain BJ5183 for DNA preparation.

The resulting transformants were then characterized by plasmid DNA isolation, BamHI restriction and agarose gel electrophoresis. One of these clones was selected for good lipase activity and for the presence of a well defined BamHI-BamHI insert of the expected size in plasmid pEX-2. This clone referred to hereinafter as pLIP2 was chosen for transformation into yeast. The nucleotide sequence of the junction between plasmid pEX-2 and the lipase-coding region was determined by the Maxam and Gilbert method and shown to be

.... GGATCCGGAGGATATTATGAAATTT...

BamHI lipase-coding region

h) Plasmid pLIP2 was used to transform the Ura3- yeast strain 01904B and the transformants screened

on minimal medium. One of the resulting clones was cultured at 28°C in liquid minimal medium till stationnary phase (about 4 days). The cells were then harvested by centrifugation, resuspended in a phosphate buffer 0.1M pH7 and disrupted by shaking with glass beads in a Braun homogeneizer. A 0.2 ml part of the resulting homogenate was used to determine lipase activity by the same radioassay as used above, taking the lipase commercialized by Germe (Marseille, France) as a standard. Total proteins were determined on another aliquot part by the method of Lowry modified by D. Herbert et al. (P.R. Stewart, Methods in Cell Biol., Ed. by P.R. Stewart Vol. XII, p. 113, 1981). It was thus determined that 17 international units of lipase (1 unit = amount of lipase bringing about the liberation of 1 mg of fatty acids per minute) corresponding to 0.342 mg of enzyme (specific activity: about 300 u/mg) were present in 20.5 mg of total proteins, i.e. the expressed lipase corresponded to 1.7% of total proteins.

Claims

fatty ac	ids contain	ing cis-9 un:	saturation, c	haracterized	e hydrolysis o I in that said ide sequence	segment of	les with a spe comprises a	cificity for sequence	15
	10	20	30	40	50	. 60	70 TGTTTGCGTTG	80 CAGCC	20
•	90	100	110	120	130	140	150 AATTTTGCGGG	160	25
AGAGCT	170 Atcccgtate	180 CTCAGGGCTGG	190 GTCGCGGGAC	200 AAGCTGTATG	210 CAGTTGATTT	220 TTGGGACAA	230 Gacaggcacaa	240 Attat	<i>30</i>
AACAAT	250 GGACCGGTAT	260 ITATCGCGATI	270 Itgtgcaaaa	280 GGTTTTAGAT	290 GAAACGGGTG	300 Gaaaaaag	310 TGGATATTGTC	320 GCTCG	
CAGCAT							390 AACGTCGTGAC		35
GCGGCG	-4-10 CGAACCGTTT	420 Igacgacaggo 500	430 Caaggegett	440 CCGGGAACAG 520	450 ATCCAAATCAA 530	460 AAAGATTTI/ 540	470 ATACACATCCA 550	480 TTTAC 560	40
AGCAGT							FCCATGGCGTT		45
	CCTTCTGTAG	CAGCAGCCAAC	STCAACAGCC	TGATTAAAGA	AGGGCTGAAC	GCGGGGGC	CAGAATACGAA	TTAA	
specific 2. Clo which	ity. oning vector catalyses th	capable of e	nsuring in a of triglyce	yeast the ex	pression of a	DNA segm	substantially to sent coding for scids contain comprising the	r a lipase ing cis-9	<i>50</i>
	de sequenc						. •	J	<i>55</i>

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	10	20	30	40	50	60	70	80
	ATGAAATTTGTAAA	RAAGAAGGAT	CATTGCACTT	GTAACAATTT	TGATGCTGTC	GTTACATCG	CTGTTTGCGT	TGCAGCC
5	90	100	110	120	130	140	150	160
	GTCAGCAAAAGCC	GCTGAACACA	ATCCAGTCGT	TATGGTTCAC	GGTATTGGAG	GGCACCATT	CAATTTTGCG	GGAACTA
10	170 AGAGCTATCCCGT/	180 Atctcagggc	190 TGGTCGCGGG	200 Acaagctgta	210 TGCAGTTGAT	220 FTTTGGGACA	230 Agacaggcac	240 AAATTAT
	250 AACAATGGACCGG	260 Tattatcgcg	270 Atttgtgcaa	280 Aaggttttag	290 Atgaaacggg		310 GTGGATATTG	320 TOGOTOG
15					•			
	330 CAGCATGGGGGGC	340 GCGAACACAC	350 TTTACTACAT	360 Aaaaatctg	370 Gacggcggaa	.380 Ataaagttgc	390 Aaacgtcgtg	400 Acgettg
	·4·10	420	430	***	450	***		
20	GCGGCGCGAACCG	420 TTTGACGACA	430 GGCAAGGCGC	440 TTCCGGGAAC	450 Agatccaaati	460 Caaaagattt	470 Tatacacatc	480 Catttac
					_*		•	
	490 Agcagtgccgata	500 TGATTGTCAT	510 Gaattactta	520 TCAAGATTAG	530 Atggtgctag	540 Aaacgttcaa	550 Atccategog	560 TTEGACA
25								, , , , , , , , , , , , , , , , , , , ,
	570 CATCGGCCTTCTG	580 Tacagcagco	590 Aagtraarag	600	610	620	630	640
	0.1.0200011010	THE ROCK OCC.	·	CCIGNIIAAA	annouge Gn	ACGGCGGGGG	CLAGARIACO	NA 1 1 NA
30								
<i>35</i>	and subfragment specificity. 3. Cloning vectorsed with a DNA yeast. 4. Cloning vectorsectors	tor according A segment co	g to claim 2 o oding for a s g to claim 3,	characterized signal sequer characterized	in that the Dince allowing to	NA segment the secretion	t coding for to the lipas	he lipase is e from said
40	chicken lysozyme 5. Cloning vec- lipase which cate unsaturation, cha nucleotide seque	e, Escherichia tors capable alyses the hy tracterized in	a coli beta-lad of ensuring drolysis of t	ctamase and in a bacteriu triglycerides	Aspergillus a m the expres with a specif	wamori gluco sion of a DN ficity for fatty	pamylase. A segment o acids conta	oding for a
45								
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<i>55</i>								
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65								

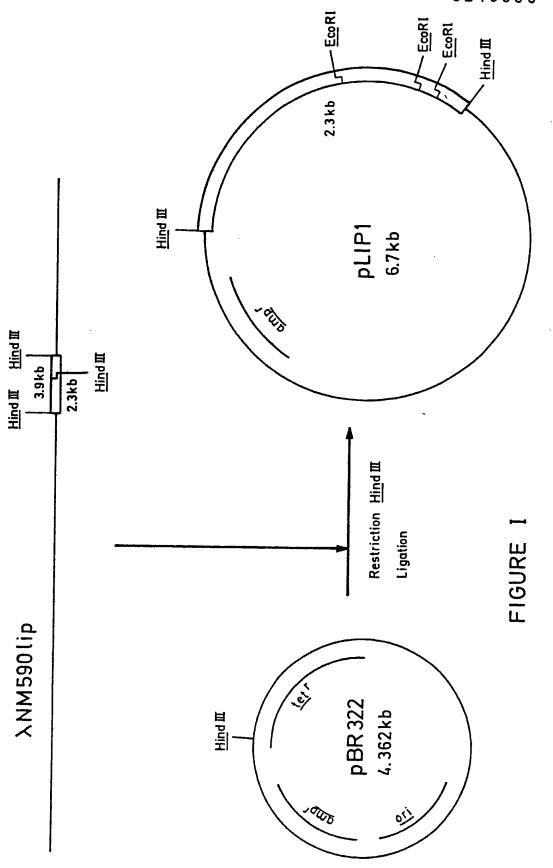
10	20	30	40	50	60	70	80	
AIGAAATIIGTA	NAAAAGAAGGATC	ATTGCACTT	GTAACAATTT	TGATGCTGTCT	TGTTACATCG	CTGTŢĪGCGT	TGCAGCC	
90	100	110	120	130	140	150	160	5
GTCAGCAAAAGC	CGCTGAACACAA	TCCAGTCGT	TATEGTTCAC	GGTATTGGAG	GGCACCATT	CAATTTTGCG	GGAACTA	
170	180	190	200	210	220	230	240	
AGAGCTATCCCG	STATCTCAGGGCT	GGTCGCGGG	ACAAGCTGTA'	FGCAGTTGAT1	TTTTGGGACA.	AGACAGGCACA	TATTAA	10
250	260	270	280	290	300	310	320	
AACAATGGACCG	GTATTATCGCGA	TTTGTGCÅA	AAGGTTTTAG	ATGAAAC GGG1	FGC GAAAAAA.	GTGGATATTGT	POTOBO	
330	340	350	360	370	380	390	400	15
CAGCATGGGGG	GCGCGAACACACT	TTACTACAT	AAAAAATCTG	GAC GGC GGAAA	TAAAGTTGC	AAACGT CGT GA	CGCTTG	
-4-10	420	430	440	450	460	470	480	
GCGGCGCGAACC	GTTTGACGACAG	GCAAGGCGC	TTCCGGGAAC	AGATCCAAATC	AAAAGATTT	TATACACATCO		20
490	500	510	520	530	540	550	560	
AGCÁGTGCCGAT	ATGATTGTCATG	AATTACTTA	TCAAGATTAG	TGGTGCTAGA	AACGTTCAA			
570	580	590	. 600	610	620	630	540	25
	GTACAGCAGCCA						640 ATŤAA	
	•				•	•		
	•						·	30
and the subfrag specificity. 6. Cloning ve	ctor according	to claim 5, c	haracterized	in that the D	NA seament	coding for th	ne linase is	
fused with a Dibacterium.	NA segment co	ding for a s	ignal sequen	ce allowing ti	he secretion	of the lipase	from said	<i>35</i>
which catalyse unsaturation, ch	s the hydrolysi naracterized in ti	s of trialyd	erides with	a specificity	for fatty ac	ids containir	na a cis-9	
nucleotide sequ	ience							40
								45
								45
								50
							,	
•								55
				•				
								60
								65

					-			
	10	20	30	40	50	60	70	80
	ATGAAATTTGTAA	AAAGAAGGAT	CATTGCACTT	GTAACAATTT	TGATECTETC	TGTTACATCG		
•	90	100	440	•				
5	GTCAGCAAAAGCC		110 Atcoagtogt	120 Tatesticas	130	140	150	160
		oo i diineneni	NICONUICUI	INIGGIICAC	GOINIIGAG	GGGCALLAII	CAATITIGEG	GGAACTA
	170	180	190	200	210	220	230	240
10	AGAGCTATCCCGT	ATCTCAGGGC	TGGTCGCGGG	ACAAGCTGTA	TGCAGTTGAT	TTTTGGGACA	AGACAGGCAC	TATTAAA
	250	260	270	200				
	AACAATGGACCGG			280 Aaggitttag	290 _. Atgaargg	300 Tergaaaaa	310 Stecatatte	320
15					MI GAAAG GOO	I UUUNAAAA	GIGGNINIIG	166166
15	330	340	350	360	370	380	390	400
	CAGCATGGGGGGC	GCGAACACAC'	TTTACTACAT	AAAAAATCTG	GACGGCGGAA	ATAAAGTTGC.	AAACGTCGTG	ACGCTTG
	-4-10	420	430	. 440	450	460		
20	GCGGCGCGAACCGT			-		460 Caaaagattt	470 Fatacacatei	480 CATTTAC
								P. 111. A.C
	490	500	510	520	530	540	550	560
25	AGCAGTGCCGATAT	TGATTGTCAT(GAATTACTTA	TCAAGATTAG	ATGGTGCTAG.	AAACGTTCAA	ATCCATEGCET	TTGGACA
	570	580	590	600	610	620	630	640
	CATCGGCCTTCTG	TACAGCAGCC						
30								
	and the subfragm	ents and mu	tants thereof	coding for i	ipases which	have retaine	d substantial	v the same
	specificity.							
<i>35</i>	8. Vector plash fused with a DNA	segment co	oding for a s	ignal sequer	nce allowing	INA segment the secretion	coding for the lipase	he lipase is e from said
	yeast.							
	9. Vector plasn group comprising	j the signal s	equence of i	nvertase, ac	id phosphata	se o-matino	factor a-ma	ed from the
40	cnicken lysozyme	e, Escherichia	a coli beta-lac	tamase and	Aspergillus a	wamori olucc	amvlaco	
70	10. Vector plasn lipase which cata	llyses the hy	drolysis of tr	ialvcerides i	with a specifi	icity for fatty	acid contain	P_ain a nie_Q
	unsaturation, cha nucleotide seque	racterized in	that said DN/	A segment is	selected from	m the group o	comprising th	e following
	ndoseoside seque	1106						
45								
50								
<i>55</i>								
~								
60								•

10 ATGAAATTTGT/	20 Aaaagaaggatc	30	40 GTAACAATTT	50 FGATGCTGTCT	60 GTTACATCG	70 	80 (GCAGC'C	
			•					
90 GTCAGCAAAAG	100 CCGCTGAACACAA	110 TCCAGTCGT	120 Tatggttcac	130 SGTATTGGAGG	140 iggcaccatt(150 Caattttgcg(160 Gaacta	5
170	180	190	200	210	220	230	240	
AGAGCTATCCC	GTATCTCAGGGCT							10
250	260	270	280	290	300	310	320	
AACAATGGACC	GGTATTATCGCGA	TTTGTGCAA	AAGGTTTTAG	ATGAAACGGGT	GCGAAAAAA	GTGGATATTGI	CGCTCG	
330	340	350	360	370	.380	390	400	15
CAGCATGGGGG	GCGCGAACACACT	TTACTACAT	AAAAAATCTG	GAC GGC GGAAA	TAAAGTTGC	AAACGTCGTGA	CGCTTG	
-4-10	420	430	440	450	460	470	480	20
GCGGCGCGAAC	CGTTTGACGACAG	GCAAGGCGC	TTCCGGGAAC	AGATCCAAATC	AAAAGATTTI	TATACACATCO	ATTTAC	20
490	500	510	520	530	540	550	560	
AGLAGIGLLGA	TATGATTGTCATG	AATTACITA	I CAAGAT TAG	ATGGTGCTAGA	AACGTTCAA	ATCCATGGCGT	TGGACA	25
570	580	590	600	610	620	630	640	
CATCEGCCTTC	TGTACAGCAGCCA	AGTCAACAG	CCTGATTAAA	SAAGGGCTGAA		CAGAATACGA	ATTAA	
								30
specificity. 11. Plasmid acwith a DNA schacterium. 12. Vector pla	gments and mut ccording to clair egment coding asmid capable o of a DNA segme	n 10 charact for a signa f autonomou	terized in tha al sequence us replication	t the DNA sec allowing the	gment codin secretion o	g for the lipa of the lipase of ensuring in	se is fused from said	<i>35</i>
specificity for	fatty acids cont he group compr	taining a cis	-9 unsaturat	ion, characte	rized in that	t said DNA s	segment is	40
								45
							•	50
								<i>55</i>
								60
								65

				0 243 330	•			
	10	20	30	40	50	60	70	80
	ATGAAATTTGTAA	AAAGAAGGAT(CATTGCACTT	GTAACAATTT	TGATGCTGTC	TGTTACATCG	CTGTTTGCGT	TGCAGCC
5	90	100	110	120	130	140	150	160
	GTCAGCAAAAGCC	GCTGAACACA	ATCCAGTCGT	TATEGTTCAC	GGTATTGGAG	GGGCACCATT	CAATTTTGCG	GGAACTA
	170	180	190	200	210	220	230	240
10	AGAGCTATCCCGT	ATCTCAGGGC	TEGTCGCGGG	ACAAGCTGTA	TGCAGTTGAT	TTTTGGGACA.	AGACAGGCAC.	
	250	260	270	280	290	300	310	320
	AACAATGGACCGG	TATTATCGCG	ATTTGTGCAA.	AAGGTTTTAG	ATGAAACGGG			
15	. 330	340	350	360	370	380	390	400
	CAGCATGGGGGGC	GCGAACACAC						
	·4·10	420	430	440	450	460	470	400
20	GCGGCGCGAACCG							480 Catttac
	490	. 500	510	520	530	540		
	AGCAGTGCCGATA			-		540 Aaacgttcaa	550 Atccatggcg	560 TTGGACA
<i>25</i>								
	570 CATCGGCCTTCTG	580 Tacagcagcca	590 Nagtcaacag	600 CCTGATTAAA	610 Gaagggetga	620 Acggcagga	630 CCAGAATACG	640
				•				101100
<i>30</i>						•		
	and the subfragm specificity. 13. Vector plasn							
<i>35</i>	of sequences fro	m the 2-mic	ron plasmid	and compris	ing at least t	he replication	n origin of th	e 2-micron
	plasmid. 14. Vector plasi	mid accordin	g to anyone	of claims 12	2 and 13 cha	racterized in	that the DN	A seament
	coding for the lip the lipase from sa	ase is fused t	with a DNA s	egment codi	ng for a sign	al sequence a	allowing the s	ecretion of
40	15. Vector plasm	nid according	to claim 14	characterize	d in that the	signal seque	nce is select	ed from the
	group comprising chicken lysozyme	e, Escherichia	a coli beta-lac	ctamase and	Aspergillus a	wamori gluco	o-amylase.	-
	16. Vector plasn pLIP2.	nid according	to anyone of	f claims 12 ar	nd 13, charac	terized in tha	t said plasmid	d is plasmid
45	17. Vector plass bacterium the ex	mid capable	of autonomo	us replicatio	n in a bacter	rium and cap	able of ensu	ring in said
	triglycerides with	a specificity	for fatty aci	ds containin	g a cis-9 uns	saturation, ch	naracterized	in that said
	DNA segment is s	selected from	i trie group c	omprising th	e tollowing nu	icleotide seq	uence	
50								
	•							

10 Atgaaatttgtaa	20 NAAGAAGGATO	30	40 GTAACAATTT	50 IGATGCTGTC	60 EGTTACATCG	70 CTGTTTGCGT	80	
			•					
90	100	110	120	130	140	150	160	5
GTCAGCAAAAGCCC	GCTGAACACAA	TCCAGTCGT	TATEGTTCAC	GGTATTGGAG	GGGCACCATT	CAATTTTGC 5:	SGAACTA	
•.								
170 AGAGCTATCCCGTA	180	190	200	210	220	230	240	
NONGCINICCO	TICICAGOCI	0010000	ACAMBCI GIA	IGLAGIIGAI	IIII OOGACA.	MUNICA GOLALA	-MAILAI	10
250	260	270	280	290	300	310	320	
AACAATGGACCGGT	TATTATCGCGA	TTTGTGCAA	AAGGTTTTAG	TGAAACGGG	TGCGAAAAA	GTGGATATTS	CGCTCG	
330	340	350	360	370	.380	390	400	15
CAGCATGGGGGGC	GCGAACACACI	TTACTACAT	AAAAAATCTG	SACGGCGGAAA	ATAAAGTTGC	AAACGTCGTG:	CSCTTG	
-4-10	420	430	440	450	460	470	480	
GCGGCGCGAACCGT								20
							•	
490	500	510	520	530	540	550	560	
AGCAGTGCCGATAT	GATTGTCAT	BATTACTTA	TÇAAGATTAG/	TGGTGCTAG	AAACGTTCAA	ATCCATGGC 57	TEGACA	
								25
570 CATCGGCCTTCTG	580	590	` 600	610	620 • • • • • • • • • • • • • • • • • • •	630	640	
CATCOSCCTICTO	IACABCABCCA		LLIGATIAAA	SARGUGU I GAA	• .		'A I I A A	
								30
								30
and the subfragm specificity.	ents and mu	tants thereof	coding for lip	pases which	have retaine	d substantiall	y the same	
18. Vector plasm	id according	to claim 17.	characterized	d in that the D	ONA seamen	t coding for t	he linase is	
fused with a DN	A segment o	oding for a	signal seque	ence allowing	g secretion	of the lipase	from said	35
bacterium.	lal according	to oleim 17 .		lim Alama and alam				
 19. Vector plasm 20. Transformed 	veast charac	terized in th	characterized at it comprise	i in that said p is a plasmid a	olasmia is pla accordina to	ismid pLIP1. anyone of cla	ims 7 8 9	
12, 13, 14, 15 and	16.					·		
21. Transformed the group compri	yeast accord	ling to claim	20 character	ized in that it	belongs to t	he genus sel	ected from	40
Candida								
22. Transformed	yeast accord	lig to claim 2	1, characteriz	ed in that it b	elongs to th	e species sel	ected from	
the group compri pombe, Kluyveron	ising Saccha nyces lactis	romyces cer Candida cylir	revisiae, Sac	charomycop	sis lipolytica,	Schizosacci	naromyces	45
23. Transformed	bacterium c	haracterized	in that it cor	nprises a pla	smid accord	ling to anyon	e of claims	45
10, 11, 17, 18 and	19.					•		
24. Transformed the group compris	oacterium at sina Escheric	coroing to d hia coli and f	ciaim 23, chai Bacillus subtil	acterized in	that said bad	terium is sel	ected from	
25. Process for	preparing a l	pase which	catalyses the	hydrolysis	of triglycerid	es with a spe	ecificity for	50
fatty acids contain					sists of growi	ing a yeast ac	cording to	
claims 20, 21 and 2 26. Process for					of trialvoerid	es with a sna	ecificity for	
fatty acids conta	ining cis-9 ι	ınsaturation,	, characterize	ed in that it	consists of	f growing a	bacterium	
according to claim	ns 23 and 24 a	ınd recoverir	ng the lipase t	hus produce	d.	-		<i>55</i>
27. Lipase obtain	eu according	to anyone o	ı cıaıms 25 ar	10 26.				



10	<u>Hin</u> e	dIII .	40	50	60	70	0.0
	ATCATCGATAAG				OU GCGAATAAGC	70 CTTCTTTTT	CB TTGGCTT
AAACTGTCGAA	TAGTAGCTATTC	GAATAAAGTT	ACTCATAACTI	CTTTTCTTC	GCTTATTCG	GAAGAAAAA	AACCGAA
90	100	110	120	130	140	150	160
AAATCCTGGTT	TAATGACCTCTG ATTACTGGAGAC	AAICIIAAAA TTAGAATTTT	IIILIIIAAAA	IATACCETTI	TAATCCCAA	ACTAATAATT	TGGTAAC
AAATCCTGGTT	AT TACTODADAC	I I AGAATI I I	AAAGAAAT I I I	INTICOUTTI	TAATGGGAA	IGATTATTAA	ACCATIG
170	180	190	200	210	220	230	240
GTAATAATT	GGAGAATTTGTT	ACAAAAAAA	GAGGATATTAT	GAAATTIGTA	LAAAAGAAGG	ATCATTGCAC	TTGTAAC
CATTATATTAA	CCTCTTAAACAA	TGTTTTTTC	CTCCTATAATA	CTTTAAACAT	TTTTCTTCC	TAGTAACGTG	AACATTG
250	260	270	280	200	200	- 0	200
	rgtctgttacat(290 'CAGCAAAGC	300 CGCTGAACA	CAATCCAGTC	320
TTAAAACTACGA	CAGACAATGTA	GCGACAAACG	CAACGTCGGCA	GTCGTTTTCG	GCGACTIGI	GTTAGGTCAG	CAATACC
330	340	350	360	370	380	390	400
TTCACGGTATT	GAGGGGCACCA	TCAATTTTG	CGGGAACTAAG	AGCTATCCCG	TATCTCAGG	GCTGGTCGCG	GGACAAG
AAGIGCCATAAC	CTCCCCGTGGT	AAGIIAAAAC	CCCIIGATIC	TCGATAGGGC	ATAGAGTCC	CGACCAGCGC	CCTGTTC
410	420	430	440	450	460	470	480
	FGATTTTTGGGA						AAAAGGT
GACATACGTCA	CTAAAAACCCT	STTCTGTCCGT	GTTTAATATT	GTTACCTGGC	CATAATAGC	GCTAAACACG	TTTTCCA
					_		
490	500	510	520	530	540	550	560
AAATCTACTTT	GGGTGCGAAAA	TTCACCTATA!	GIUGUIUGUA MAGAGAGAGA	CCTACCECCC	CGCGAACAC.	ACTITACTAC	AAAAA TATTTT
70011011101111	CCCACGCTTTT	ITONCOTATAL	CAUCUAUCUI	OGINGOCCC	000017010		
570	580	590	600	610	620	630	640
570 ATCTGGACGGC	580 GGAAATAAAGTT(590 GCAAACGTCG1	600 GACGCTTGGC	610 GGCGCGAACC	620 GTTTGACGAI	630 CAGGCAAGGC	640 SCTTCCG
570 ATCTGGACGGC	580	590 GCAAACGTCG1	600 GACGCTTGGC	610 GGCGCGAACC	620 GTTTGACGAI	630 CAGGCAAGGC	640 SCTTCCG
570 ATCTGGACGGCC TAGACCTGCCGC	580 GGAAATAAAGTTO CCTTTATTTCAAO	590 GCAAACGTCG1 CGTTTGCAGC <i>A</i>	600 GACGCTTGGC ACTGCGAACCG	610 GGCGCGAACC CCGCGCTTGG	620 GTTTGACGA(CAAACTGCT(630 CAGGCAAGGC GTCCGTTCCG	640 GCTTCCG CGAAGGC
570 ATCTGGACGGCC TAGACCTGCCGC	580 GGAAATAAAGTTO CCTTTATTTCAAO 660	590 GCAAACGTCG1 CGTTTGCAGCA 670	600 GACGCTTGGC ACTGCGAACCG 680	610 GGCGCGAACC CCGCGCTTGG	620 GTTTGACGA(CAAACTGCT)	630 CAGGCAAGGC GTCCGTTCCG	640 GCTTCCG CGAAGGC 720
570 ATCTGGACGGCC TAGACCTGCCGC	580 GGAAATAAAGTTO CCTTTATTTCAAO 660 MAATCAAAAGATT	590 GCAAACGTCG1 CGTTTGCAGCA 670 FTTATACACA1	600 GACGCTTGGC ACTGCGAACCG 680 CCCATTTACAG	610 GGCGCGAACC CCGCGCTTGG 690 CAGTGCCGAT	620 GTTTGACGA(CAAACTGCT) 700 ATGATTGTC	630 CAGGCAAGGC GTCCGTTCCG 710 ATGAATTACT	640 GCTTCCG CGAAGGC 720 TATCAAG
570 ATCTGGACGGCC TAGACCTGCCGC 650 GGAACAGATCCA CCTTGTCTAGGT	580 GGAAATAAAGTTO CCTITATTTCAAO 660 MAATCAAAAGATT TTAGTTTTCTAA	590 GCAAACGTCGT CGTTTGCAGCA 670 FTTATACACAT AAATATGTGTA	600 GACGCTTGGC ACTGCGAACCG 680 CCCATTTACAG AGGTAAATGTC	610 GGCGCGAACC CCGCGCTTGG 690 CAGTGCCGAT GTCACGGCTA	620 GTTTGACGA(CAAACTGCT) 700 ATGATTGTC/ TACTAACAG	630 CAGGCAAGGC GTCCGTTCCG 710 ATGAATTACT TACTTAATGAA	640 GCTTCCG CGAAGGC 720 FATCAAG ATAGTTC
570 ATCTGGACGGCC TAGACCTGCCGC 650 GGAACAGATCCA CCTTGTCTAGGT	580 GGAAATAAAGTTO CCTTTATTTCAAO 660 MAATCAAAAGATT TTTAGTTTTCTAA	590 GCAAACGTCGT CGTTTGCAGCA 670 ITTATACACAT AAATATGTGTA 750	600 GACGCTTGGC ACTGCGAACCG 680 CCCATTTACAG AGGTAAATGTC	610 GGCGCGAACC CCGCGCTTGG 690 CAGTGCCGAT GTCACGGCTA	620 GTTTGACGAI CAAACTGCT 700 ATGATTGTC/ TACTAACAG	630 CAGGCAAGGC GTCCGTTCCG 710 ATGAATTACT FACTTAATGAA	640 GCTTCCG CGAAGGC 720 TATCAAG ATAGTTC
570 ATCTGGACGGCC TAGACCTGCCGC 650 GGAACAGATCCA CCTTGTCTAGGT 730 ATTAGATGGTGC	580 GGAAATAAAGTTO CCTTTATTTCAAC 660 MAATCAAAAAGATT TTAGTTTTCTAA 740 CTAGAAAACGTTCA	590 GCAAACGTCGT CGTTTGCAGCA 670 FTTATACACAT AAATATGTGTA 750 AAATCCATGGC	600 GACGCTTGGC ACTGCGAACCG 680 CCCATTTACAG AGGTAAATGTC 760 AGTTGGACACA	610 GGCGCGAACC CCGCGCTTGG 690 CAGTGCCGAT GTCACGGCTA 770 TCGGCCTTCT	620 GTTTGACGAI CAAACTGCT 700 ATGATTGTC/ TACTAACAG 780 GTACAGCAG	630 CAGGCAAGGC GTCCGTTCCGC 710 ATGAATTACT FACTTAATGAA 790 CCAAGTCAAC	640 GCTTCCG CGAAGGC 720 FATCAAG ATAGTTC 800 AGCCTGA
570 ATCTGGACGGCC TAGACCTGCCGC 650 GGAACAGATCCA CCTTGTCTAGGT	580 GGAAATAAAGTTO CCTTTATTTCAAC 660 MAATCAAAAAGATT TTAGTTTTCTAA 740 CTAGAAAACGTTCA	590 GCAAACGTCGT CGTTTGCAGCA 670 FTTATACACAT AAATATGTGTA 750 AAATCCATGGC	600 GACGCTTGGC ACTGCGAACCG 680 CCCATTTACAG AGGTAAATGTC 760 AGTTGGACACA	610 GGCGCGAACC CCGCGCTTGG 690 CAGTGCCGAT GTCACGGCTA 770 TCGGCCTTCT	620 GTTTGACGAI CAAACTGCT 700 ATGATTGTC/ TACTAACAG 780 GTACAGCAG	630 CAGGCAAGGC GTCCGTTCCGC 710 ATGAATTACT FACTTAATGAA 790 CCAAGTCAAC	640 GCTTCCG CGAAGGC 720 FATCAAG ATAGTTC 800 AGCCTGA
570 ATCTGGACGGCC TAGACCTGCCGC 650 GGAACAGATCCA CCTTGTCTAGGT 730 ATTAGATGGTGC TAATCTACCACG	580 GGAAATAAAGTTO CCTTTATTTCAAC 660 MAATCAAAAAGATT TTAGTTTTCTAA 740 CTAGAAACGTTCA SATCTTTGCAAGT	590 SCAAACGTCGT CGTTTGCAGCA 670 ITTATACACAT AAATATGTGTA 750 AAATCCATGGC ITTAGGTACCG	600 GACGCTTGGC 680 CCATTTACAG GGTAAATGTC 760 GGTTGGACACA	610 GGCGCGAACC CCGCGCTTGG 690 CAGTGCCGAT GTCACGGCTA 770 TCGGCCTTCT AGCCGGAAGA	620 GTTTGACGAI CAAACTGCT 700 ATGATTGTC/ TACTAACAG 780 GTACAGCAG CATGTCGTC	630 CAGGCAAGGCG GTCCGTTCCGG 710 ATGAATTACT FACTTAATGAA 790 CCAAGTCAACAGGTCAACAGGTCAACAGTCAACAGTCAACAGTCAACAGTCAACAGGTCAACAGTTCAGTTGGAACA	640 GCTTCCG CGAAGGC 720 FATCAAG ATAGTTC 800 AGCCTGA FCGGACT
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CAATCAGCATCAATATGGCGACAATAATGACACCGGCGGACCCATCGCCAAACGTAAATCCGGCTGCCAAAATCACTGCT GTTAGTCGTAGTTATACCGCTGTTATTACTGTGGCCGCCTGGGTAGCGGTTTGCATTTAGGCCGACGGTTTTAGTGACGA GCGGCAATGATTACATCAAGTAAAAGAGCGCAGGCGCGCAGCATGAAACCAGCTAGTTCCAATAGAAACACTCCTTAAAA CGCCGTTACTAATGTAGTTCATTTTCTCGCGTCCGCGCGTCGTACTTTGGTCGATCAAGGTTATCTTTGTGAGGAATTTT TGTTAAATAAACACCTAATGATTGTAAAAAAGAAGGGCCAAAGTGGGAATAGGTGATAAGCCTTAAATCACAAAAGTTGG ACAATTTATTTGTGGATTACTAACATTTTTTCTTCCCGGTTTCACCCTTATCCACTATTCGGAATTTAGTGTTTTCAACC CGAAAATGCCATAGGTAAA "TGGCATAATCAGCCAGCTTATCACATTACCAAATTCTTTTTTAGCCCGAAACCAAGCCCT GCTTTTACGGTATCCATTTAACCGTATTAGTCGGTCGAATAGTGTAATGGTTTAAGAAAAAATCGGGCTTTGGTTCGGGA EcoRI CAGAAGTTATTTTTGTTAAAATAGAAAAGTTACAACAGAATTCGGAGGGTTTATTGTGGGAAAAGCGAAACGAAATGCCC GTCTTCAATAAAAACAATTTTATCTTTTCAATGTTGTCTTAAGCCTCCCAAATAACACCCTTTTCGCTTTGCTTTACGGG CTTGCCCATGCGGCAGCGGCAAGAAATATAAAAAATGCTGCGGAAGTAAAGTTGTCGACTTCCCGGCGGAACTAGCGGCA GAACGGGTACGCCGTCGCCGTTCTTTATATTTTTTACGACGCCTTCATTTCAACAGCTGAAGGGCCGCCTTGATCGCCGT AAAGAAGCGAAACAAATTCAGGAAGACTTAGTGGAGTATGCCTTCACAGTACATAGAGAAAGCATTTCAGGCTTTATCAA TTTCTTCGCTTTGTTTAAGTCCTTCTGAATCACCTCATACGGAAGTGTCATGTATCTCTTTCGTAAAGTCCGAAATAGTT CCAGCATGATTTTCTTTCTGCTATGGACAGACAGACGAAAGACATCAGCGTATTTAACTTAGGAATCTGGGGAATCCTCC GGTCGTACTAAAAGAAAGACGATACCTGTCTGTCTGCTTTCTGTAGTCGCATAAATTGAATCCTTAGACCCCTTAGGAGG TCCACCCGCTTGCTGGTGAGAAGACAGTCTTCGAAGAGTACCTTCGGAAAAAAGGCGATTCGATCACTCGA

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